p53 and EGFR Expression in Glioblastoma Multiforme

- *An Immunohistochemical Study*

Dissertation submitted to the Dr. M.G.R. Medical University, Chennai, for the part III M.Ch. Neurosurgery Examination, August 2007

CERTIFICATE

This is to certify that the following thesis is a bonafide record of the study done by Dr. C. Livingston in part fulfillment of the requirement for M.Ch. Neurosurgery course under the guidance of Dr. Geeta Chacko, Professor, Division of Neuropathology with the help of Dr. Ari. G. Chacko, Professor & Head of Neurosurgery, Unit I and Dr. V. Rajshekhar, Professor & Head of Neurosurgery Unit II.

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Introduction

Over the last twenty years, with the advances in molecular biology, the understanding of tumorigenesis has reached a stage where novel treatments are being experimented. The present study analyzes the expression pattern of p53 and EGFR in glioblastoma multiforme and correlates this to outcome.

Literature Review

In 1863 Virchow described Glioblastoma multiforme (GBM) as a highly malignant brain tumor of glial origin with the worst prognosis of all the brain tumors[17]. These tumors are notorious for their rapid invasion into neighboring structures of brain along the white matter, perivascular space, subependymal plane or subpial plane[5, 7]. Glioblastoma multiforme accounts for 12-15% of intracranial neoplasms[26] and 50-60 % of the astrocytic tumors[52]. The peak incidence is between 45-70 years[52], but they also occur in children[9]. Males are more commonly affected than females[52]. The commonest location is in the cerebral subcortical white matter in the following order of frequency - temporal lobe, parietal lobe, frontal lobe and occipital lobe. The typical histological features include nuclear atypia, microvascular proliferation, multinucleated giant cells and necrosis[17]. In the WHO classification it has been allocated a “grade 4” owing to its highly malignant and bizarre characteristics.

WHO classification:

WHO classification [17, 19] is more widely accepted than the previously used Kernohan [16] and Ringertz [37] grading of astrocytomas. The St.Anne/Mayo grading system, based on four criteria (nuclear atypia, mitosis, microvascular proliferation and or necrosis) is however both reproducible and predictive of patient survival. Comparison of the WHO grading with the St. Anne/Mayo grading is given in Table I. As a general rule the grading is based on the area of highest degree of anaplasia, with the assumption that this tumor cell population eventually determines the course of the disease. Apart from the histopathological features, the patient’s survival also depends on age, Karnofsky’s performance score, tumor location and treatment. The average survival pattern is more than five years for diffuse astrocytoma (WHO grade II), two to five years for anaplastic astrocytoma (WHO grade III) and less than one year for glioblastoma (WHO grade IV).

Table 1: Comparison of WHO grading of Astrocytoma with the St

O WHO Grade	WHO designation	St.Anne/Mayo designation	Histological criteria
I	Pilocytic astrocytoma		
II	Diffuse astrocytoma	Astrocytoma grade 2	One criterion, usually nuclear atypia
III	Anaplastic astrocytoma	Astrocytoma grade 3	Two criteria, usually nuclear atypia and mitotic activity
IV	Glioblastoma multiforme	Astrocytoma grade 4	Three criteria: nuclear atypia, mitoses, endothelial proliferation and/or necrosis

Anne/Mayo grading system.

In 1940 Scherer described two different types of GBMs, primary GBMs and secondary GBMs[38]. Primary GBMs were those with a shorter duration of symptoms usually less than 3 months, seen in adults older than 45 years and with a bad prognosis. Secondary GBMs were those that progressed from a low grade tumor through to a high grade astrocytoma, with a longer duration of symptoms, seen in younger patients usually less than 45 years of age [15, 31].

Ohgaki et al[32] in their study of 715 cases of GBM concluded that 38 patients had clinical and histopathological evidence of progression from a less malignant precursor lesion and these were diagnosed as secondary GBMs. The remaining 677 patients showed either clinical or histopathological evidence of highly malignant lesion and were classified as primary GBMs.

At present it is increasingly accepted that the two types of GBMs have different genetic pathways of evolution[18, 32]. The primary GBMs are supposed to arise from deregulation of epidermal growth factor receptor pathway[15, 16] and the secondary GBMs are supposed to arise from deregulation of cell cycle pathway by mutation of p53 gene which is a tumor suppressor gene or over expression of p53 protein[48, 49]. Lang et al[25] in his study of 65 astrocytic gliomas postulated different genetic pathways leading to GBMs. One pathway was characterized by 43 astrocytomas with alteration in p53. GBMs with p53 alterations

represented tumors that progressed from low grade astrocytoma. This variant was more likely to show loss of chromosome 17p than tumors without p53 alterations ($p<0.04$). Seventy five percent of tumors with loss of one 17 p allele demonstrated mutation in the *p53* gene. Loss of chromosome 10 was associated with progression from anaplastic astrocytoma (13%) to glioblastoma (38%) ($p<0.04$). Amplification of EGFR gene was a rare (7%) but late event in tumor progression ($p<0.03$). A second pathway was characterized by six astrocytomas without p53 alterations and represented clinically denovo high grade tumors. These tumors were more likely to show amplification of EGFR gene (83%). 60% of them also showed loss of chromosome 10; loss of chromosome 17p was infrequent in this variant. In addition, there were 16 astrocytomas with none of the known genetic changes. He concluded that GBMs were a heterogeneous group of tumors that probably arose via multiple genetic pathways.

Hayashi et al[12], in their study of 70 patients of GBM noted that EGFR amplification and *p53* mutation were mutually exclusive ($p<0.0001$, Fisher exact test). They also noticed that *p53* mutation was seen in younger patients and EGFR amplification was seen in older patients and tumors with EGFR amplification behave in a more aggressive manner. Watanabe et al[51] in their study also found that *p53* and EGFR mutations were exclusive for secondary and primary GBMs respectively.

Recent reports revealed that the survival after surgery, radiotherapy and chemotherapy is better in secondary GBM compared to primary GBM.

Cell growth and proliferation are enhanced by 'proto-oncogenes' such as, the epidermal growth factor receptor gene, whereas genes which restrict abnormal cell division and growth are termed 'tumor suppressor genes' such as, *p53*.

p53 is a tumor suppressor gene located on the short arm of chromosome 17p.13.3. The protein is primarily a sequence specific transcriptional activator. It binds to responsive elements within the genome and activates the transcription of genes residing in the vicinity of these binding sites. The proteins encoded by *p53* target genes, whose number is probably in the hundreds, contribute in multiple ways to the biological effects of p53. The biological outcome of p53 activity includes apoptosis, inhibition of cell cycle, senescence, differentiation and accelerated cell repair. *p53* is believed to reside in a biologically latent state

in the absence of cellular stress, although the exact biochemical nature of this latency is under debate. When the cells experience a variety of stress conditions, *p53* becomes activated. Activation involves a marked increase in cellular p53, as well as qualitative changes that endow each p53 molecule with improved capabilities to modulate gene expression and alter the cell phenotype. The types of stress that promote *p53* activation include many conditions associated with cancer initiation and progression, such as direct DNA damage, chromosomal aberration, illegitimate activation of oncogenes, hypoxia, telomere shortening and more[33]. *p53* expression is studied by quantifying the p53 protein by immunohistochemical studies by biotin avidin peroxidase method and *p53* gene mutation is identified by studying loss of heterozygosity, southern blot analysis[28] .

EGFR

Epidermal growth factor receptor (EGFR) is a transmembrane receptor that binds to extracellular ligands such as epidermal growth factor and transforming growth factor alpha and transduces mitotic signals[1]. It has an external ligand binding site, a trans membrane component and an intracellular cytoplasmic region which modulates signal transduction pathways by activation of kinase pathway. EGFR amplification has been identified as a genetic hallmark of GBMs. The predictive value of EGFR amplification has been unclear. Layfield et al[27], have noted that EGFR identification by immunohistochemistry correlates well with identification of EGFR gene amplification by fluorescence in situ hybridization (FISH).

Factors affecting glioblastoma multiforme prognosis:

Age

Pre operative Karnofsky's performance score

Location of the lesion

Extent of the excision

Histopathology

a) Giant cells

b) Gemistocytes

c) Oligodendroglial component

d) Cystic component

di)

Radiation

Chemotherapy

Genetic factors

Age:

Patients with younger age have a better prognosis compared to the older patients. It has also been found that secondary GBMs are seen in younger patients whereas primary GBMs are seen in the older age group. Shrieve et al[42], in their study of 78 patients with GBM reported that patients less than 40 years of age have a median survival time of 48.6 months compared to patients older than 40 years who's median survival time was 18.2 months ($p<0.0001$). Korshunov et al[21] and Lamborn et al[24] also reported an age < 40 years as an important prognostic indicator of better survival. Ohgaki et al[32] in his study also showed by both univariate and multivariate analyses that old age is a significant predictor of poor survival in GBMs. The cut off age in his series was 50 years, with younger patients (<50 years) having a significantly longer survival (median 8.8 months) than older patients (>=50 years; median, 4.1 months; $p<0.0001$). Sneed et al[45] in their study on 159 glioblastoma multiforme patients noticed that nine patients between 18 and 29.9 years of age had a 3 years survival probability of 78+/- 14%. The 64 patients between 30 and 49.9 years of age had a 3 year survival probability of 29+/-6% and 86 patients >= 50 years had a 3 years survival probability of 6+/-3%. Stark et al[46], in their study of 267 cases of GBM noted that age below 61 years was significantly associated with prolonged survival ($p<.001$).

Pre Operative Karnofsky's performance status score:

Karnofsky's performance score is a good prognostic indicator. Layfield et al[27] in their study of 34 patient of glioblastoma multiforme noted that those with a higher Karnofsky's performance status score survived longer. Stark et al[46], in their study of 267 patients of GBM showed that a pre operative Karnofsky's performance status score of 70 or more was a good prognostic indicator ($p<.001$). Lacroix et al[23] in their

study of 416 patients with GBM also reported a better prognosis with good Karnofsky's performance status score.

Duration of symptoms:

It is believed that patients with primary GBM have a shorter duration of symptoms, usually less than three months, compared to patients with secondary GBM It has also been reported that patients with a shorter duration of symptoms have a worse prognosis[2].

Location of lesion:

Superficial lesions have a better prognosis compared to the deep seated lesions. This is due to the wider excision that can be performed on superficial lesions. Lacroix et al[23], in their study of 416 patients of GBM observed that tumors located in the eloquent brain were associated with a shorter duration of survival but this effect was lost in multivariate analysis. He did not find a survival difference between deeply located tumors and superficial tumors.

Extent of excision:

Patients who have a radical excision have a better prognosis compared to the patients who have a partial excision or biopsy.. Lacroix et al[23], in their study of 416 patients with GBM found that there was a significant survival advantage associated with resection of 98% or more of the tumor volume, median survival was 13 months compared with 8.8 months for resection less than 98% ($p<0.0001$). Kreth et al[22], in their study of 57 patients reported a median survival time for resection followed by radiotherapy group of 39.5 weeks as compared with 32 weeks for biopsy followed by radiotherapy group. This difference was however not statistically significant. Barker et al[4], in their study of 301 patients with GBM found that the extent of resection and the immediate response to radiation therapy correlated with survival both in univariate analysis and multivariate Cox model after correction for age and KPS ($p<0.0001$ for radiation response and $p=0.04$ for extent of resection). Stark et al[46], in their study of 267 cases of GBM also reported that total tumor excision was significantly associated with prolonged survival ($p=.014$).

Histopathology of the lesion:

Giant cell:

Homma et al[13], in their study of 403 patients of GBM showed that GBMs containing ≥ 5 % multinucleated cells had a poorer survival. However, patients with giant cell glioblastoma had a longer survival (12.4±16.2 months) than those with other forms of GBM (8.4±-7.9 months), but this difference was not significant. (p=0.105). Shinojima et al[41], in their study of 113 patients of GBM six patients survived more than five years. Incidentally three of the six were diagnosed as giant cell GBM.

Gemistocytes:

Homma et al[13], in their study had found no correlation between the presence of gemistocytes and prognosis. When present in large numbers, particularly in a patient known to have a pre existing glioma, these cells may represent a lower grade precursor lesion within a secondary glioblastoma. Reis et al[36], in their study mention that gemistocytic variants of low grade astrocytoma are prone for rapid progression to anaplastic astrocytoma and GBM.

Oligodendroglial component:

Oligodendroglial component is present in about 20% of GBMs[13]. Patients with GBM containing an oligodendroglial component are significantly younger[13]. In a study by Homma et al[13], the median survival of GBM patients with an oligodendroglial component was 8.1 months and for those without an oligodendroglial component it was 6 months. Pinto et al[34], in their study on 47 cases of GBM also detected a longer survival in patients with an oligodendroglial component. Vordermark D et al[50], in their study of 10 cases of GBM with an oligodendroglial component showed that these patients had a better response to chemotherapy and radiation therapy compared to those without this component.

Cystic component

Maldaun et al[29], in their study of 22 cases of cystic GBM found that the median survival time after surgery was 18.2 months and that at 2 years 43 % of the patients were still alive. In patients with non cystic GBM the median survival time was 14.3 months and only 16 % of patients were alive at 2 years. The median time for tumor recurrence was 7.6 months in patients harboring cystic GBMs and 4.2 months in the non cystic GBM group. Though there was a trend towards better prognosis for cystic GBMs there was no statistical significance.

Radiation therapy:

At present multimodality therapy is favored for GBM which includes surgery, radiotherapy and chemotherapy. Barker et al[3], studied 301 GBM patients and concluded that younger patients (p=0.006), high pre operative Karnofsky's performance score (p=0.027) and more extensive surgical resection (p=0.028) predicted better radiation response in univariate analyses. Almost similar responses were noted in multivariate analyses. They had in an earlier study also reported similar findings.[27]. Fazeny et al[10] in their study of 98 GBM patients noted that the median survival time for patients who underwent only stereotactic biopsy was 9 weeks, 13 weeks for patients who underwent biopsy and radiotherapy and 31 weeks for patient who received radiation and chemotherapy($p<=0.001$) following biopsy.

Chemotherapy:

Chemotherapy improves the survival marginally when combined with surgery and radiation. Combs et al[8], in their study on 53 patients diagnosed to have GBM concluded that the overall survival is relatively long for patients who had radiation therapy and chemotherapy compared to the survival times reported for radiation alone. Fine et al[11], in a meta analysis of 16 randomized clinical trials, which included more than 3000 patients treated between 1975 and 1989, demonstrated a survival benefits for those patients with malignant glioma treated with radiation and adjuvant chemotherapy compared to those treated with radiation alone. Stupp et al[47] in their study have shown that addition of temozolomide to radiation therapy confers a meaningful survival advantage compared with post operative RT alone in GBM. As mentioned previously Fazeny et al[10] has shown that patients with chemotherapy and radiation therapy survived better compared to patients who underwent biopsy alone and patients who underwent biopsy and radiation therapy ($P= 0.001$). Mirimanoff et al[30] in their study of five hundred and seventy three patients subjected 219 patients for postoperative radiotherapy and chemotherapy with a median survival time of 15 months. 261 patients who underwent radiation therapy alone had a median survival time of 12 months.

Genetic factors:

There are various genetic factors considered to affect the prognosis of patients with GBM. There are conflicting reports on the prognostic influence of *p53* mutation or over expression and EGFR amplification

or over expression. Kleinschmidt et al[20], in their study of 38 patients with GBM observed that there was a shorter patient survival with extensive necrosis, extensive expression of p53 and absence of EGFR amplification. None of these factors however reached statistical significance. Homma et al[13] in their study of 420 cases of GBM correlated key genetic alteration and clinical outcome. EGFR amplification and p16 homozygous deletion were significantly more frequent in small cell glioblastomas than in non small cell glioblastomas. Multivariate analysis with adjustment for age and gender showed that small cell glioblastoma had frequent EGFR amplification and p16 deletion but infrequent PTEN mutations. Simmon et al[43], and Smith et al[44] showed that there was no association between p53 expression and outcome in patients with GBM. Schmidt et al[39], in their study of 97 patients suggested that p53 expression was a favorable prognostic indicator of GBM (p=0.0085). Ohgaki et al[32], in their population based study suggested that *p53* mutation was predictive of favorable prognosis in univariate analysis. Birner et al[6] in their study of 114 patients of primary GBM noted that there were 29 patients with p53 protein expression. These patients were significantly younger and had a significantly longer survival in univariate analysis (p=0.0399, log rank test). Reavey-Cantwell et al[33] in their study of 32 patients of glioblastoma on the other hand concluded that there was no prognostic significance of p53 expression.

EGFR amplification has been associated with poorer survival in patients with GBM[14, 51]. Shinojima et al[40], reported that EGFR amplification was a unfavorable predictor for overall survival in glioblastoma patients but that the EGFR gene status was a more significant prognostic factor in younger patients (<60 years). Simmons et al[43] , also reported that EGFR over expression was associated with poorer survival of GBM patients younger than the median age and that EGFR over expression was negatively associated with survival in cases without TP 53 mutation. Smith et al[44] on the other hand, reported that EGFR amplification was a predictor of longer survival only in older glioblastoma patients, However, Ohgaki et al[32], in their study on 715 cases of GBM showed that there was no correlation between EGFR amplification and survival at any age. Schmidt et al[39] in their study of 97 patients also showed a lack of predictive value of EGFR amplification. This was further corroborated by a meta-analysis of seven previous studies which did not detect a significant predictive value of EGFR amplification[15].

Aims and Objectives

1. To determine the expression pattern of p53 and EGFR in glioblastoma multiforme.

2. To determine whether the above two markers predict the biological behavior of glioblastoma multiforme.

Hypotheses

1) Younger patients show p53 over expression and older patients show EGFR over expression (age >= 50 yrs versus < 50yrs).
2) EGFR is over expressed in patients with shorter duration of symptoms and p53 is over expressed in patients with longer duration of symptoms. (≤ 3months versus >3 months)
3) Males are more likely to have EGFR overexpression and females are more likely to have p53 overexpression.
4) EGFR expression is associated with poorer survival compared to p53 expression.
5) Co expression of p53 and EGFR are associated with poorer survival as compared to lack of expression of both p53 and EGFR.

Material and methods

In a prospective study, fifty eight patients who were diagnosed with GBM from June 2003 to Sept 2004 were included. Age, gender and duration of symptoms were recorded in the data sheet. The clinical symptoms were grouped as seizures, focal neurological deficits and features of raised intracranial pressure. Pre operative Karnofsky's performance status score and post operative Karnofsky's performance status score were noted. All patients had pre operative CT scan or MRI of the brain. The location of the lesion was noted. The size of the lesion was measured in centimeters. The presence of necrosis and mass effect was also noted.

Operative details:

All patients underwent open excision except one who underwent a stereotactic biopsy. The surgery was classified as radical excision, subtotal excision, partial excision and biopsy based on the post operative imaging (contrast CT or contrast MRI). Radical excision was defined as gross total removal of tumor. Sub total excision was defined as excision of 90% of the tumor and partial excision was defined as removal of less than 90% of tumor and in biopsy as those where tumor tissue sampled for histological diagnosis.

Treatment protocol:

As mentioned above, all patients underwent open surgery with the exception of one patient who had stereotactic biopsy. This was followed by radiation therapy in the form of conventional radiation or conformal radiation therapy. 180 cGy was given five days a week for a period of 31 days over 6 weeks to a total dose of 5580 cGy. All patients were given chemotherapy with CCNU or Temozolomide or PCV. CCNU was given at a dose of 200 mg/m2 in single dose every six weeks for six cycles and Temozolomide(TMZ) was given at a dose of 75mg/m2 daily concomitantly during RT and adjuvant TMZ was given at 150-250 mg/m^2 in gradually increasing doses for 5days every 28 days for 6 cycles. PCV was given for patients with glioblastoma with oligodendroglial component who were not responsive to temozolomide.

Follow-up:

Follow up was obtained either through patient record of direct visits to the clinic or through letters. Follow up was obtained in 34 patients.

Histopathology and immunohistochemistry:

All the cases were reviewed histopathologically and the diagnosis of Glioblastoma multiforme, WHO grade IV was confirmed. A representative section was chosen in each case and immunohistochemistry for p53 protein and EGFR protein was done by the avidin biotin peroxidase method using monoclonal antibodies to these two proteins. p53 (DAKO ppts) and EGFR (DAKO ppts). The protocol used in given in Appendix I. Negative and positive controls were included. p53 was considered positive if there was evidence of nuclear staining and EGFR positivity was seen as diffuse membrane staining.

Statistical method:

The data was analyzed using cross tabulations. Survival analysis was done using Kaplan Meier graphs in patients with follow up.

Results

There were 58 cases of GBM treated in this hospital that were included in this study (Fig 1&2 and Table 2)

Fig 1: Shows a gadolinium enhancing lesion of the dominant frontal lobe with extension into the ventricles and the frontal opercula.

Fig 2: Post operative scan of the same patient showing radical excision with no tumor residue. This patient did not have speech deficit post operatively.

TABLE 2. Data of all 58 study patients.

Sno	EGFR	P53 status	Follow up duration	Sex	Age	Preop KPS	Chemo	RT	dur-symp
1	+	+	17	M	56	90	TMZ	Y	2
2	+	+	NA	M	42	30	NA	NA	2
36	-	+	21	M	62	70	CCNU	Y	3
3	+	+	17	M	37	60	NA	Y	5
37	-	+	NA	M	35	80	NA	NA	5
4	+	+	20	F	35	80	NA	Y	5
38	-	+	4	M	43	60	NA	Y	3
5	+	+	NA	M	67	60	NA	NA	8
39	-	+	5	M	60	80	NA	Y	5
6	+	+	NA	F	28	70	NA	NA	27
40	-	+	20	F	35	70	CCNU	Y	5
7	+	+	11	M	55	80	TMZ	Y	10 days
41	-	+	10	M	38	70	CCNU	Y	6
8	+	-	NA	F	53	60	NA	NA	1
42	-	+	16	F	13	90	CCNU	Y	12
9	+	-	3	M	55	60	NA	Y	2
43	-	+	NA	M	35	60	NA	NA	18
10	+	-	13	M	50	70	CCNU	Y	2
44	-	+	NA	F	49	60	CCNU	NA	15 days
11	+	-	16	F	51	70	CCNU	Y	2
45	-	+	3	M	7	80	CCNU	Y	7days
12	+	-	NA	M	46	80	NA	NA	2
46	-	-	3	F	59	80	CCNU	Y	1
13	+	-	NA	M	44	50	NA	NA	2
47	-	-	10	M	55	80	TMZ	Y	1
14	+	-	17	F	30	60	CCNU	Y	3
48	-	-	NA	M	35	30	NA	NA	1
15	+	-	18	M	55	70	NA	Y	3
49	-	-	22	M	31	80	TMZ/CCNU/PCV	Y	2
16	+	-	NA	M	35	90	CCNU	Y	3
50	-	-	NA	M	60	80	NA	NA	2
17	+	-	NA	M	42	80	NA	NA	3
51	-	-	NA	F	45	60	NA	NA	3
18	+	-	24	F	37	80	NA	Y	4
52	-	-	24	M	32	70	CCNU/PCV	Y	3
19	+	-	NA	M	38	70	NA	NA	5
53	-	-	12	M	45	70	CCNU	Y	4
20	+	-	4	M	38	80	TMZ	Y	6
54	-	-	NA	M	17	50	NA	NA	6
21	+	-	10	F	55	50	TMZ	Y	19
55	-	-	10	M	55	80	NA	Y	24
22	+	-	NA	M	48	60	NA	NA	20 days
56	-	-	NA	M	68	70	NA	NA	12 days
23	+	-	6	M	54	60	TMZ	Y	5days
57	-	-	5	F	55	60	CCNU	Y	14 days
24	-	+	16	M	52	70	TMZ	Y	1
58	-	-	NA	M	25	50	NA	NA	14 days
25	-	+	NA	M	28	90	NA	NA	1
26	-	+	5	M	65	70	CCNU	Y	2
27	-	+	10	M	35	60	CCNU/PCV	Y	2
28	-	+	23	M	59	80	CCNU	Y	2
29	-	+	32	M	23	100	CCNU	Y	2
30	-	+	NA	M	27	60	NA	NA	2
31	-	+	NA	M	37	60	NA	NA	2
32	-	+	NA	M	36	70	NA	NA	2
33	-	+	NA	F	40	60	NA	NA	2
34	-	+	9	M	33	90	NA	Y	3
35	-	+	6	M	28	80	NA	Y	3

NA - Not Available ; CCNU – Carmustine; PCV – Procarbazine, Carmustine, Vincristine; TMZ – Temozolomide

Histopathology of GBM:

All 58 cases showed the classical histological features of a glioblastoma multiforme, which included mitotic activity, necrosis and endothelial proliferation (Fig 3-7).

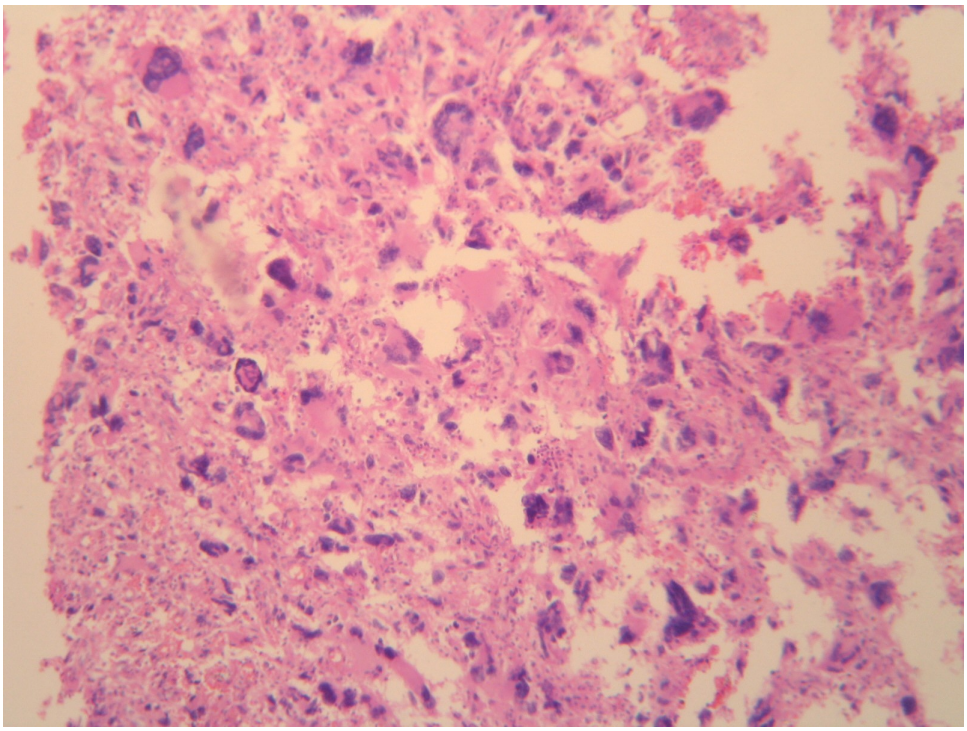


Fig 3

Glioblastoma multiforme with marked nuclear atypia, bizarre nuclei and mitotic figures (H&E X 90).

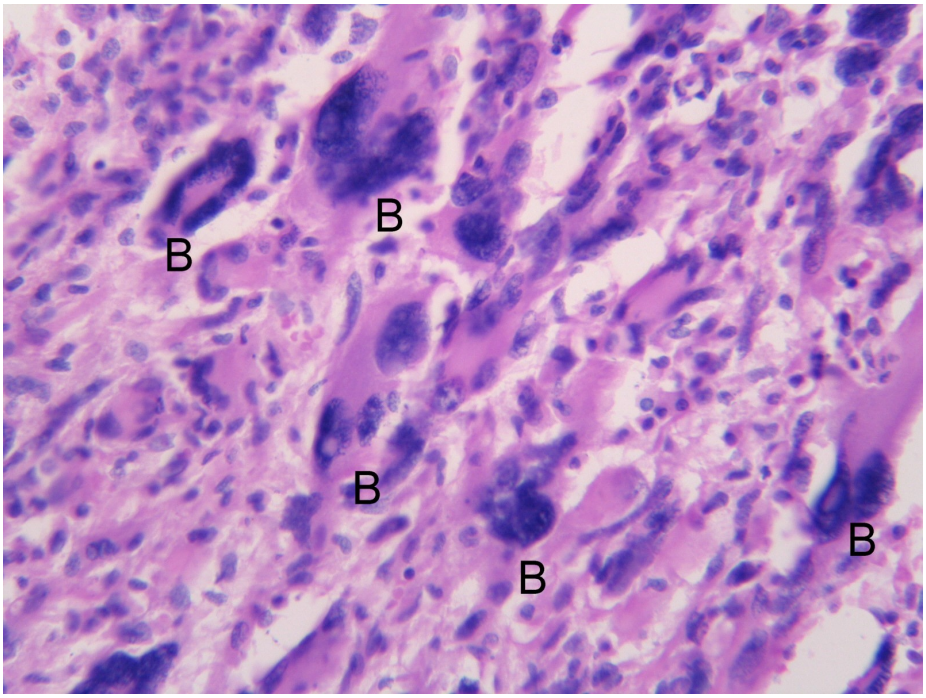


Fig 4

Glioblastoma multiforme with multinucleated giant cells and bizarre forms (B) (H&E X400).

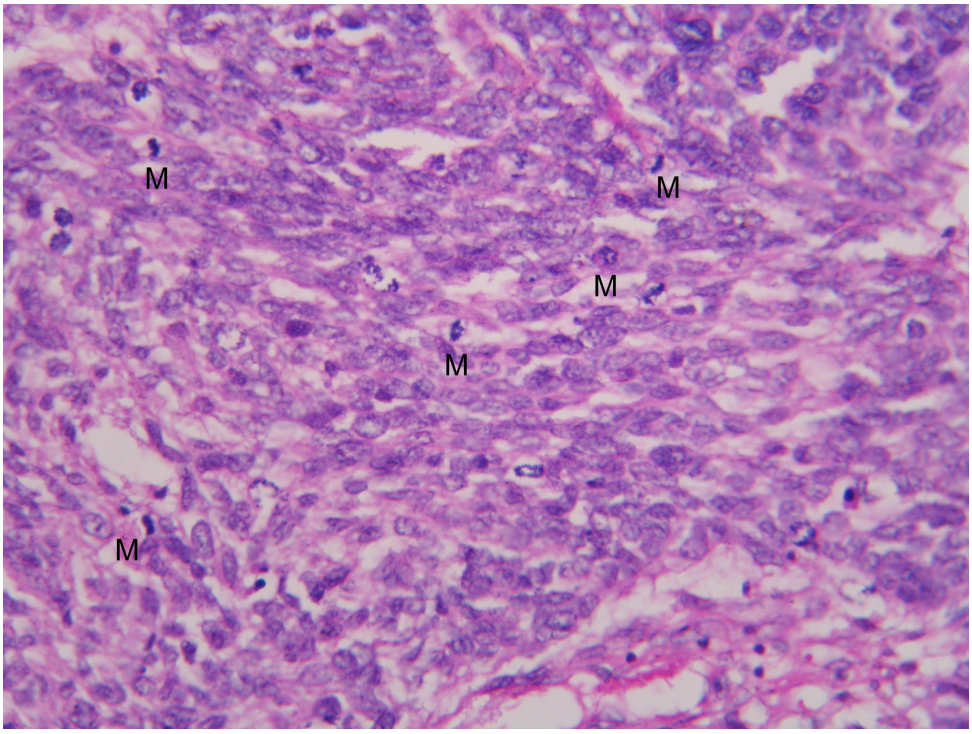


Fig 5

Glioblastoma multiforme with numerous mitotic figures (M) (H&E X400).

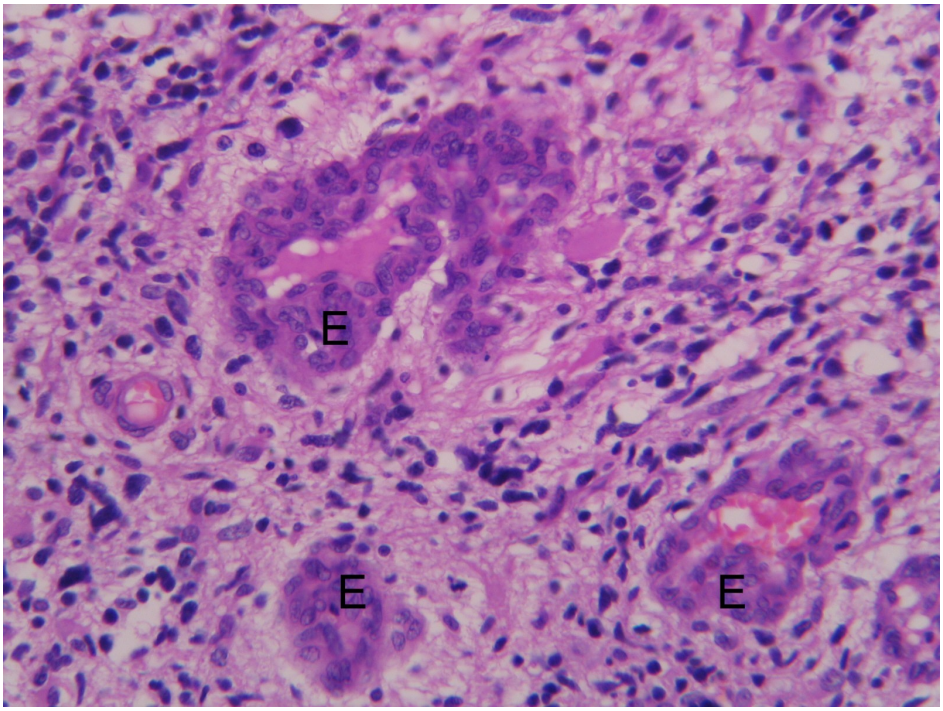


Fig 6

Vascular (endothelial) proliferation(E) in glioblastoma multiforme (H&E X400).

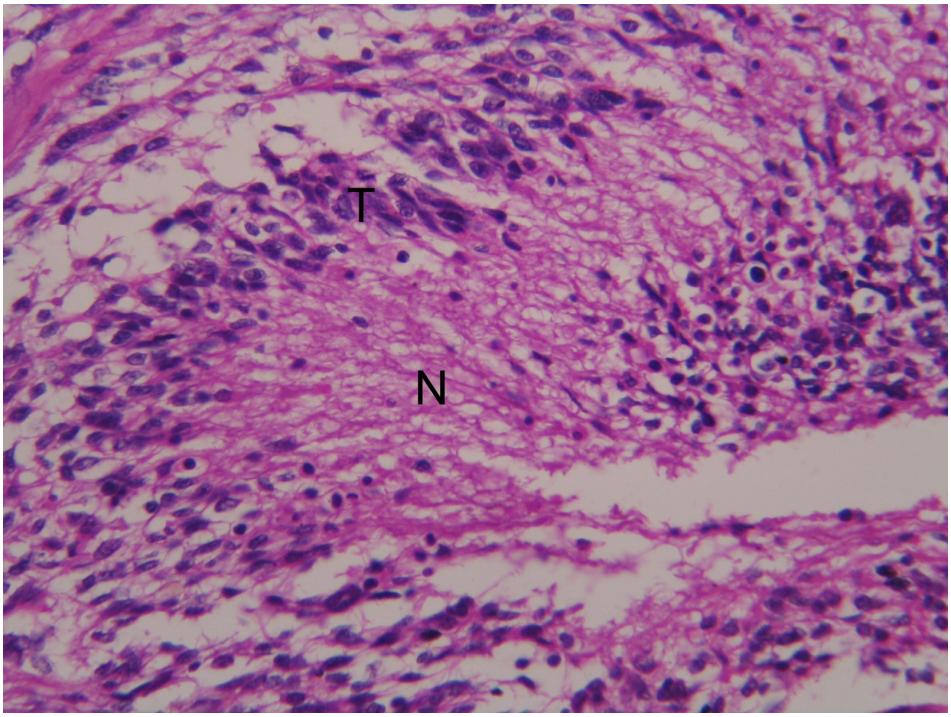


Fig 7

Palisading necrosis (N) in glioblastoma multiforme (H&E X 400).

Immunohistochemistry revealed that 7 cases were positive for both EGFR and p53, 16 were EGFR positive and p53 negative, 22 were EGFR negative and p53 positive and 13 were negative for both EGFR and p53 (Fig 8&9).

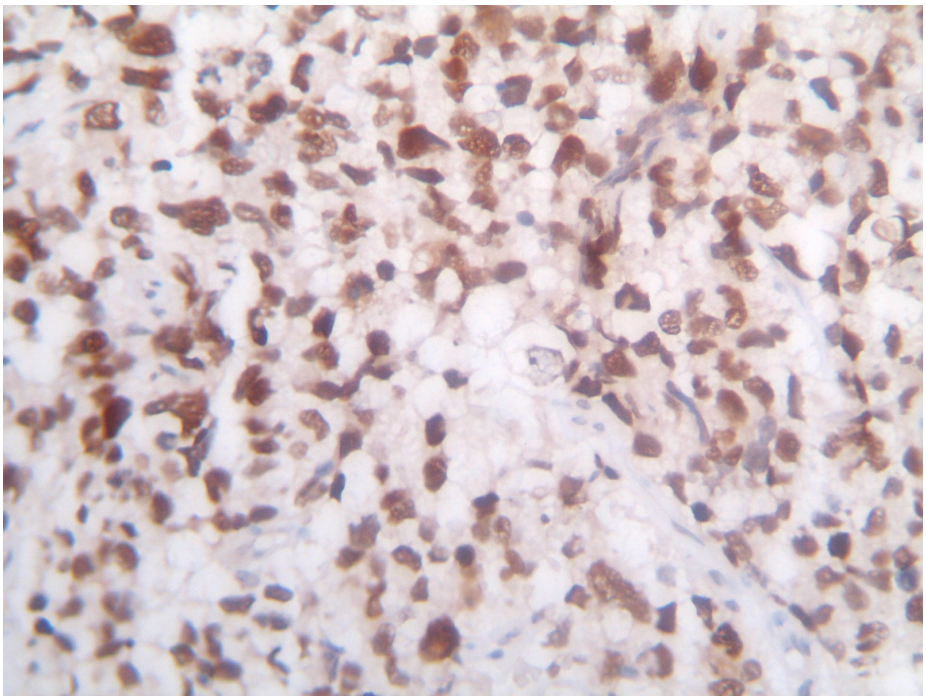


Fig 8a - Nuclear positivity for p53(P) on immunohistochemistry in cases of glioblastoma multiforme (400X).

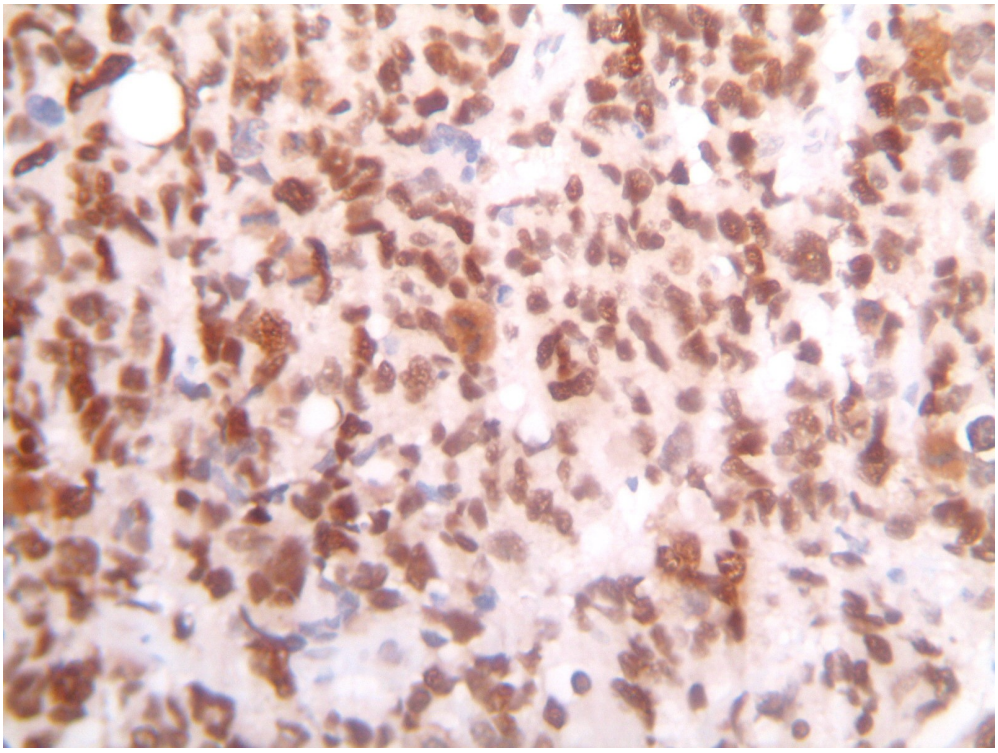


Fig 8b - Nuclear positivity for p53 (P) on immunohistochemistry in cases of glioblastoma multiforme (400X).

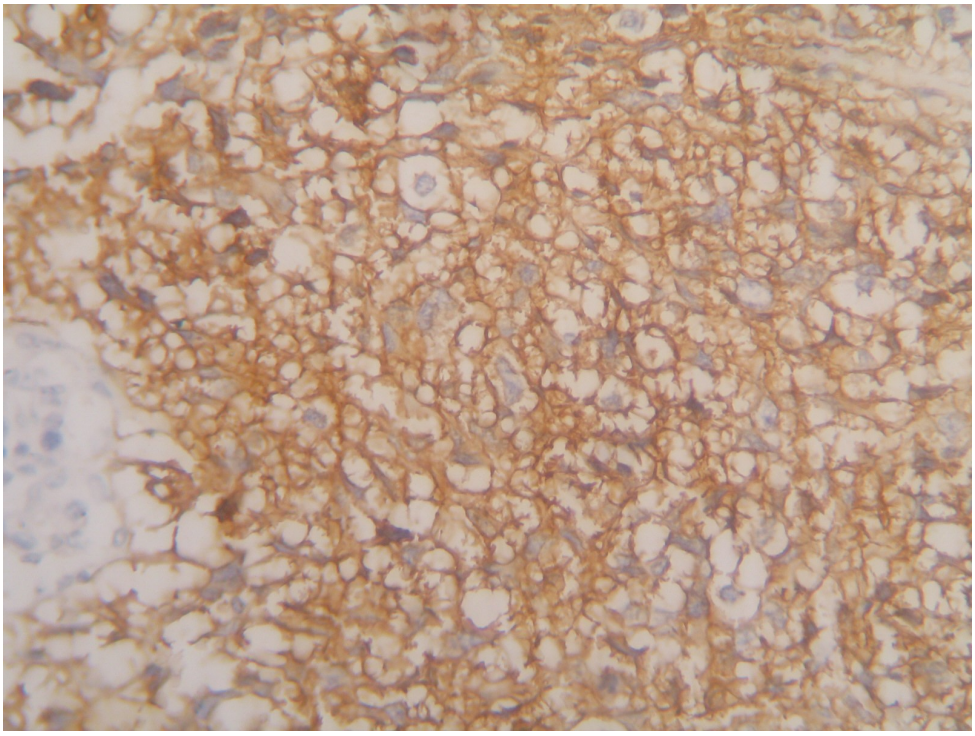


Fig 9a - Strong cytoplasmic membrane staining (P) in cases of glioblastoma with EGFR overexpression (400X)

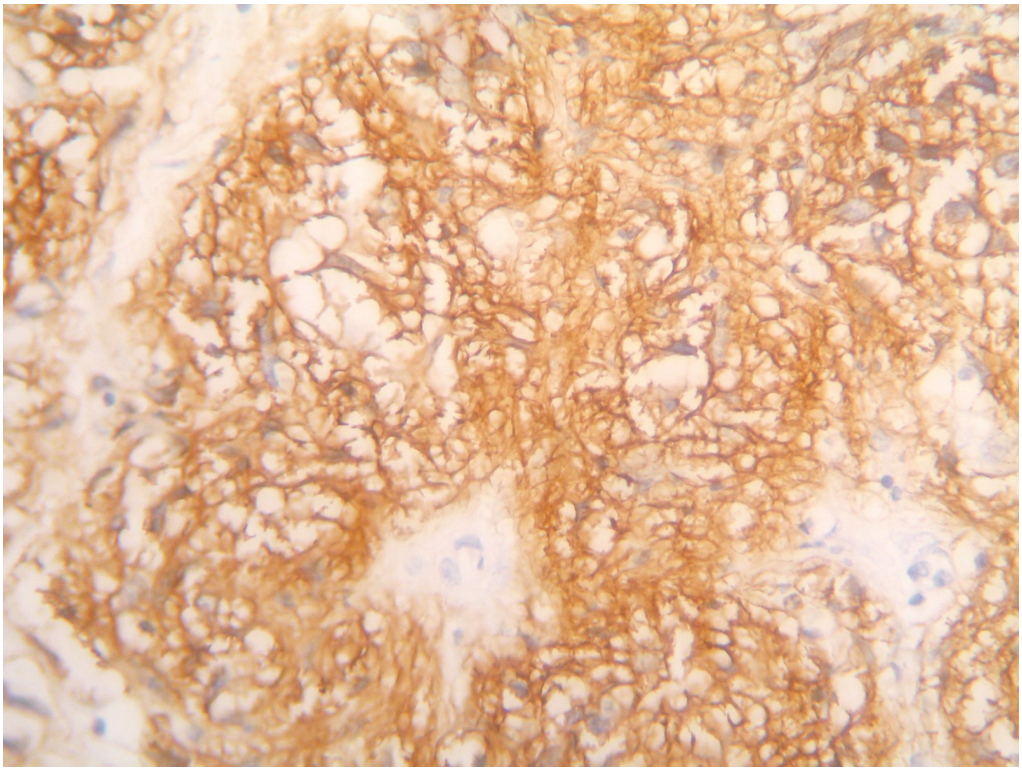


Fig 9b - Strong cytoplasmic membrane staining (P) in cases of glioblastoma with EGFR overexpression (400X).

TABLE 3. Data of the 34 patients with follow up details.

Sl no	EGFR	p53 status	Survival (months)	Sex	Age	Preop KPS	Chemo	RT	Duration of symp	Death/recurred/no recurrence
1	+	+	17	M	37	60	NA	Y	5	Died
2	+	+	20	F	35	80	NA	Y	5	Died
3	+	+	17	M	56	90	TMZ	Y	2	Died
4	+	+	12	M	55	80	TMZ	Y	10 days	Died
5	+	-	13	M	50	70	CCNU	Y	2	Died
6	+	-	3	M	55	60		Y	2	Died
7	+	-	8	M	54	60	TMZ	Y	5 days	Died
8	+	-	17	F	30	60	CCNU	Y	3	Died
9	+	-	24	F	37	80	NA	Y	4	Died
10	+	-	10	F	55	50	TMZ	Y	12	Died
11	+	-	16	F	51	70	CCNU	Y	2	Died
12	+	-	4	M	38	80	TMZ	Y	6	Recurred
13	+	-	18	M	55	70	NA	Y	3	Recurred
14	-	+	4	M	65	70	CCNU	Y	2	Died
15	-	+	21	M	62	70	CCNU	Y	3	Died
16	-	+	16	M	52	70	TMZ	Y	1	Died
17	-	+	5	M	60	80	NA	Y	5	Died
18	-	+	4	M	43	60	NA	Y	5	Died
19	-	+	20	F	35	70	CCNU	Y	5	No recurrence
20	-	+	23	M	59	80	CCNU	Y	2	No recurrence
21	-	+	16	F	13	90	CCNU	Y	12	No recurrence
22	-	+	10	M	28	80	NA	Y	3	No recurrence
23	-	+	10	M	38	70	CCNU	Y	6	No recurrence
24	-	+	32	M	23	100	CCNU	Y	2	No recurrence
25	-	+	13	M	33	90	NA	Y	3	Recurred
26	-	+	10	M	35	60	CCNU/PCV	Y	2	Recurred
27	-	+	3	M	7	80	CCNU	Y	7 days	Recurred
28	-	-	3	F	59	80	CCNU	Y	1	Died
29	-	-	10	M	55	80	TMZ	Y	1	No recurrence
30	-	-	24	M	28	70	CCNU/PCV	Y	3	Recurred
31	-	-	10	M	55	80	NA	Y	24	Recurred
32	-	-	22	M	31	80	TMZ/CCNU/PCV	Y	2	Recurred
33	-	-	6	F	55	60	CCNU	Y	14 days	Recurred
34	-	-	12	M	45	70	CCNU	Y	4	Recurred

Hypothesis 1:

Younger patients (≤ 50yrs) show p53 overexpression and older patients (> 50 yrs) show EGFR over expression

It was found that there was a significant association of p53 overexpression with patients age of ≤ 50 yrs (p=0.01) (Table 4). In older patients of age >50 yrs there was however no significant difference in the expression of p53 or EGFR (p= 0.39) (Table 5).

TABLE 4: p53 and EGFR expression in patients below 50 years of age (n=37).

		P53	
		+	-
EGFR	+	**4**	**9**
	-	**17**	**7**

P= 0.01; Odds ratio- 0.81; CI - 0.03, 0.97

TABLE 5: p53 and EGFR expression in patients above 50 years of age.(n=21)

		P53	
		+	-
	+	**3**	**7**
EGFR	-	**5**	**6**

P= 0.39; Odds ratio – 0.51; CI – 0.06,4.29

Hypothesis 2:

EGFR is over expressed in patients with shorter duration of symptoms (≤3months) and p53 is over expressed in patients with longer duration of symptoms (>3 months)

The present study suggests that p53 is over expressed in patients with shorter duration of symptoms (p=0.005) (Table 6) There is no significant over expression of p53 or EGFR in patients with longer duration of symptoms (p=0.34). (Table 7)

TABLE 6: p53 and EGFR expression in patients with less than 3 months of symptoms. (n=33)

		P53	
		+	-
EGFR	+	**2**	**10**
	-	**14**	**7**

P = 0.005; Odds ratio – 0.10; CI - 0.01- 0.72

TABLE 7: p53 and EGFR expression in patients with more than 3 months of symptoms. (n=25)

		P53	
		+	-
EGFR	+	**5**	**6**
	-	**9**	**5**

P= 0.34; Odds ratio – 0.46; CI – 0.07, 3.05

Hypothesis 3:

Males are more likely to have EGFR over expression and females are more likely to have p53 overexpression.

In this study p53 overexpression was significantly associated with the male patients. (p=0.003) Table 8. The female patients did not have a significant predilection for either p53 or EGFR over expression (p=0.28), Table 9.

TABLE 8: p53 and EGFR expression in male patients (n=44).

		P53	
		+	-
EGFR	+	**5**	**11**
	-	**18**	**10**

P=0.03; Odds ratio – 0.25; CI – 0.05, 1.11

TABLE 9: p53 and EGFR expression in female patients. (n=14)

		P53	
		+	-
EGFR	+	**2**	**5**
	-	**4**	**3**

P=0.28; Odds ratio – 0.30; CI – 0.02, 4.29.

Hypothesis 4:

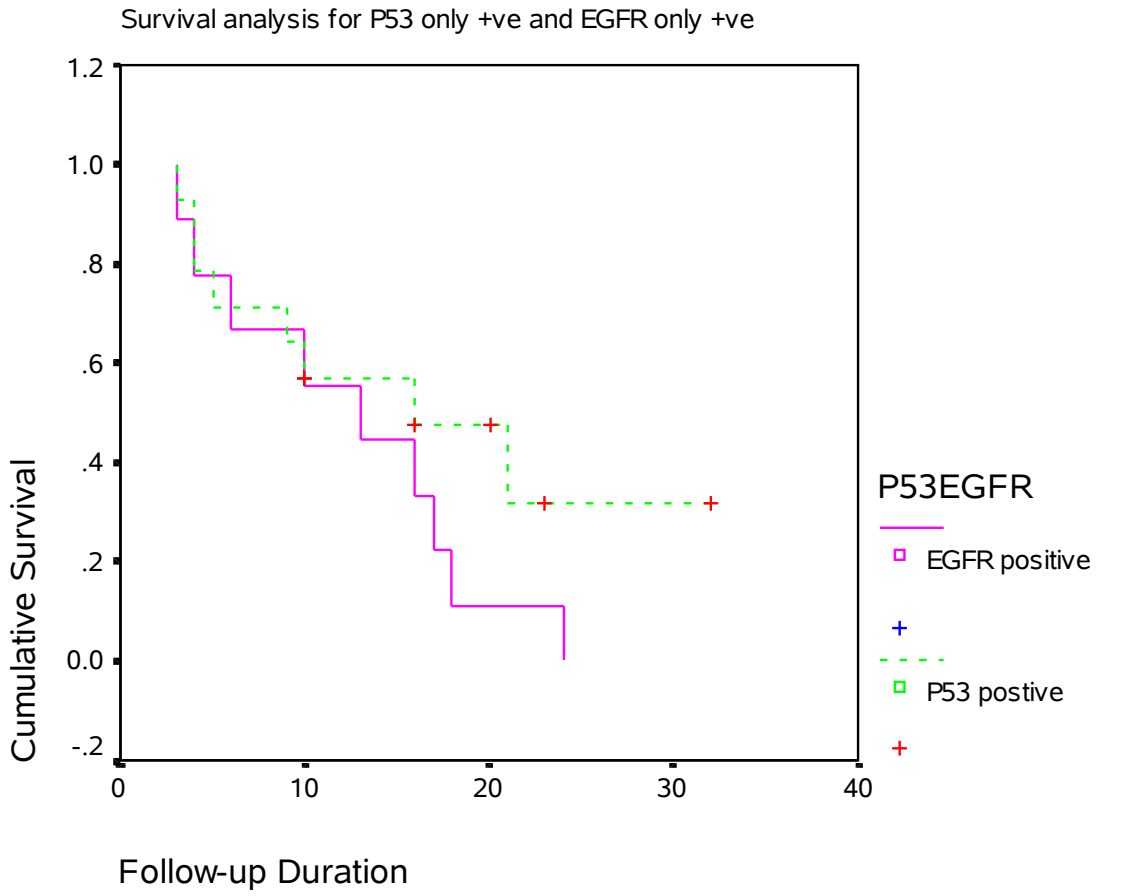
EGFR expression is associated with poorer survival compared to p53 expression.

Although patients with EGFR over expression had a shorter overall survival than patients with p53

overexpression, this was not statistically significant (p=0.39).

GRAPH 1: Survival pattern of patients with tumors that are only p53 positive or only EGFR positive .

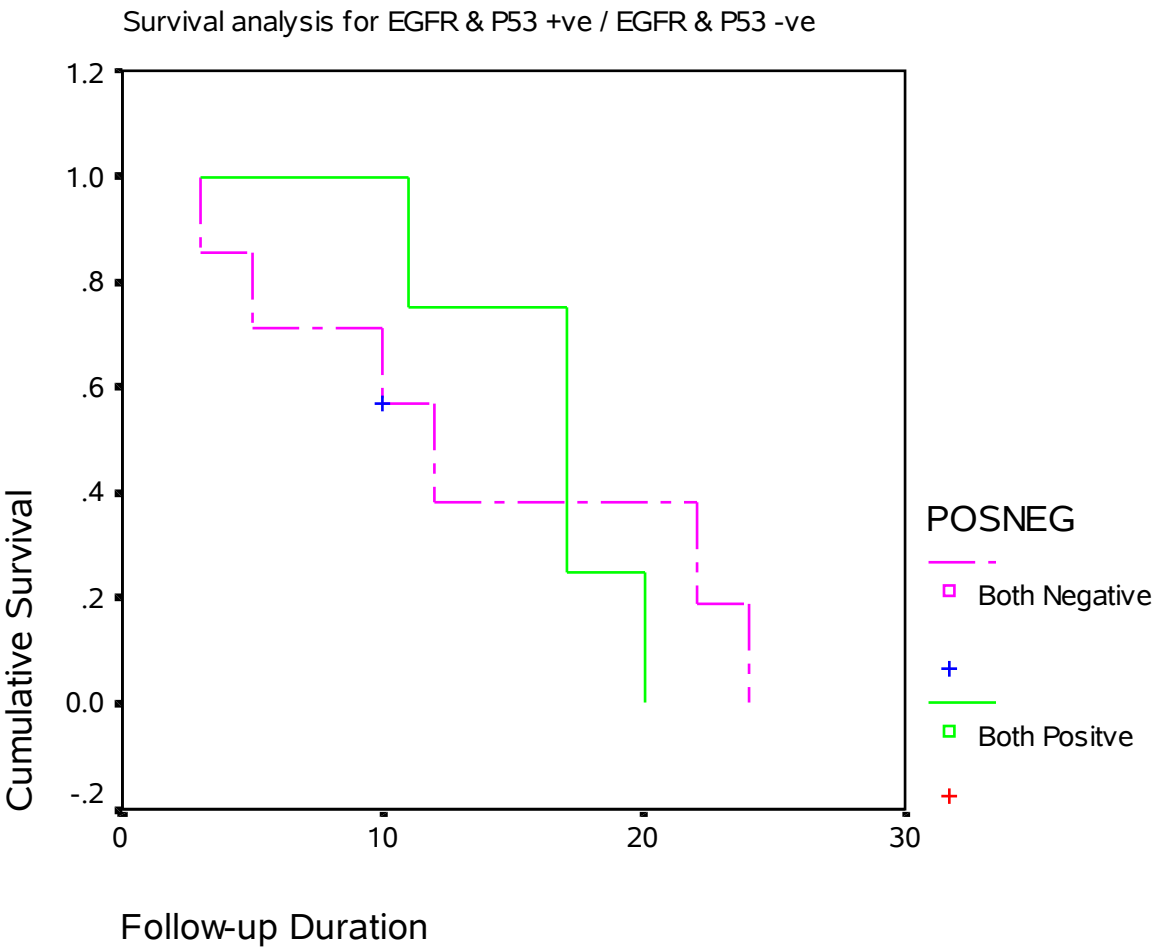
Hypothesis 5:



Co expression of both p53 and EGFR are associated with poorer survival as compared to lack of expression of both p53 and EGFR.

Although patients with both p53 overexpression and EGFR over expression had a shorter duration of survival this was not statistically significant.(p=0.79).

GRAPH 2: Survival pattern of patients with p53 & EGFR positive and p53 & EGFR negative patients.


Survival analysis for EGFR & P53 +ve / EGFR & P53 -ve
Cumulative Survival
1.2
1.0
.8
.6
.4
.2
0.0
-.2
0
10
20
30
Follow-up Duration
POSNEG
Both Negative
Both Positve

Discussion

Glioblastoma multiforme (GBM) is notorious for its rapid growth and local invasion. The main stay of the current treatment modality is radical excision followed by radiation therapy and chemotherapy. With all the available treatment modalities the expected mean survival time is 12 months. The various factors that influence the prognosis of GBM are age of the patient, pre operative Karnofsky's performance score, location of the lesion, size of the lesion, extent of excision, histological type, radiation therapy and chemotherapy. Further primary GBMs (de novo) has a poorer prognosis compared to secondary GBMs (evolved from low grade astrocytoma).

The concept of primary and secondary GBM was first observed by Scherer, a German pathologist in 1940[38]. Initially it was based on duration of clinical symptoms. As the understanding evolved there were attempts to confirm this concept with histopathological and genetic correlations. The last two decades saw an exhaustive attempt in identifying the genetic alteration underlying primary and secondary glioblastoma.

There are two different genetic pathways attributed to the development of primary and secondary glioblastomas. The EGFR pathway for primary glioblastomas and p53 pathway for secondary glioblastomas which was proposed by Kleihues et al in 1999[18] . p53 is a cell cycle regulator protein which directly regulates cell cycle by activating transcription and EGFR is a epidermal growth factor receptor protein which indirectly regulates cell cycle through the kinase pathway.

In the present study we compared the p53 and EGFR expression with age of the patient, duration of symptoms and gender. We also analyzed the survival pattern of GBM patients in various genetic groups.

Primary GBMs are generally associated with EGFR amplification and are seen in older patients, on the contrary secondary GBMs are seen in younger patients and are more often associated with *p53* mutations[5].

Of the 58 patients 33 (56.89%) patients had symptoms less than three months and 25 (43.10%) patients had symptoms more than three months. There were 21(36.20%) patients who were more than 50 years of age and 37 (63.79%) patients who were less than 50 years of age.

It is said that primary glioblastoma are seen in patients with a shorter duration of symptoms and secondary glioblastomas are seen in patients with longer duration of symptoms[53]. If duration of symptom is considered an indicator of the tumor being a primary or secondary glioblastoma, then in this study the finding of the patients with shorter duration of symptoms being p53 positive, contradicts the general theory that primary GBMs usually associated with EGFR over expression, have a shorter duration of symptoms. There was no association of longer duration of symptoms with p53 and EGFR expression.

In the present study p53 over expression was significantly associated with patient age less than 50 years. However an age of > 50 years was not associated with p53 or EGFR over expression. Watanabe et al[51] in their study showed 55 years as the mean age of primary GBMs and 39 years as the mean age of secondary GBMs. In the present study the mean age of patients with EGFR over expression was 45.2 years and the mean age of patients with p53 over expression was 38.86 years.

Louis DN et al[28] have shown that secondary GBMs are more frequently seen in women. In this study the reverse was found true with significant number of patients with p53 overexpression in the male group.

On comparing the survival pattern for patients with either p53 overexpression or EGFR over expression, although patients with EGFR over expression had a shorter overall survival than patients with p53 overexpression, this was not statistically significant. The mean survival duration was 12 months. Survival analysis of patients who were both p53 and EGFR positive and both p53 and EGFR negative, also showed no significant difference in survival duration, although patients with both p53 overexpression and EGFR over expression had a shorter duration of survival.

The findings in the present study are in keeping with those reported by Simmon et al[44], Smith et al[44] and Reavey-Cantwell et al[35] who concluded that there is no correlation between the p53 expression and

prognosis. It is however contradictory to that of Schmidt et al[39], Ohgaki et al[32] and Birner et al[6] who found that p53 over expression is a favorable prognostic indicator.

Similarly, although Hurtt MR et al[14], Torp SH et al[49], Shinojima et al[40] found that EGFR amplification was associated with poorer prognosis, the result of the present study is similar to the meta analysis of Huncharek M et al[15] and Ohgaki et al[32] which showed no association between EGFR amplification and prognosis.

As mentioned above although not statistically significant it was found in this study that patients with both p53 and EGFR over expression survived for a shorter period of time as compared to those with both genetic factors negative. The present study had fifty eight patients with follow up information available on a little over 60% of patients. Further studies on a larger cohort of patients are indicated to conclusively determine whether p53 overexpression and EGFR over expression affect prognosis in patients with glioblastoma multiforme.

Conclusions

1. There was a significant association of p53 overexpression with patient's age of < 50 yrs. In older patients of age >50 yrs there was, however, no significant difference in the expression of p53 or EGFR.

2. Contrary to the second hypothesis p53 overexpression is seen in patients with a shorter duration of symptoms (≤ 3 months) and there is no correlation between a longer duration of symptoms (>3months) and p53 or EGFR expression.

3. There were a significant number of male patients who were p53 positive. This contradicts the third hypothesis.

4. There was no significant survival difference between patients with either p53 or EGFR over expression.

5. There was no significant difference in survival between patients with both p53 & EGFR positive compared to patients with both p53 & EGFR negative.

The present study's attempt to delineate between primary and secondary GBMs based on genetic alterations based on the existing literature and analytical knowledge showed that there is no definite correlation to categorically classify GBMs to redefine the treatment protocol.

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Appendix 1

Peroxidase conjugate avidin method for paraffin sections.

1) Sections are cut at approximately 5 microns, floated on poly L-lysine coated slides & left at 37 degree overnight.
2) Do not allow slides to dry at any stage of procedure.
3) Carry out the steps of incubation with antibody in AC room.
4) Use appropriate control for each antibodies tested.

Procedure:

1) Dewax section in xylene and bring to water.
2) Transfer those sections which do not need trypsinisation to dish containing TRIS buffered saline (pH7.6) (TBS)
3) Trypsinisation
    a) Drain off tap water
    b) Transfer into preheated dish containing distilled water in 37 degree water bath.
    c) Leave for 10 min to attain temperature.
    d) Transfer into preheated trypsin solution at 37degree. Allow digestion was 10 min.
    e) Stop digestion by running tap water for 5min and then transfer to TBS.
4) Rinse twice in TBS – 5 min each
5) Drain and cover slides with
    a) Normal human pooled serum diluted1/5 monoclonal
    b) Normal swine serum diluted for 1/5 for polyclonal.
    c) Incubate for 10min.
6) Drain and cover section with optimally diluted primary antibody. Incubate for 30 min.
7) Rinse in TBS three times each 5 mins.
8) Drain and cover sections in optimally diluted second layer antibody. Incubate for 30 min.

a) Polyclonal- biotinylated swine antirabbit-dilution 1/100

b) Monoclonal- biotinylated rabbit antimouse-dilution 1/200

9) Rinse in TBS three times 5 min each

10) Block endogenous peroxidase with 0.5%H2O2 in methanol

11) Rinse in TBS three times –3 times 5mins each

12) Drain and cover slides with peroxidase conjugated avidin 1/200. Incubate for 30 min.

13) Rinse in TBS three times 5 min each.

14) Develop sections with freshly prepared diaminobenzidine solution containing H2O2 for approximately 10 min. Check positive control to ascertain end incubation.

15) Counter stain with Harris hemotoxylin- approx 10 min.

16) Dehydrate clear and mount in gum dammar.